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Ca^{2+} influx mediated through the GPIIb/IIIa complex during platelet activation

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When aequorin-loaded platelets were stimulated with thrombin, the luminescence signal of aequorin showed two peaks. From experiments with 1 mM external Ca^{2+} or EGTA, both one-half of the first peak and the entire second peak reflected the influx of Ca^{2+} from the external medium, and the remaining half of the first peak reflected the mobilization of Ca^{2+} from its storage site. A monoclonal antibody (TM83) that recognizes the glycoprotein IIb/IIIa (GPIIb/IIIa) complex which has binding sites for fibrinogen and the synthetic peptide GRGDSP are known to inhibit fibrinogen binding and platelet aggregation. Both eliminated the second peak of intracellular free calcium ($[Ca^{2+}]_i$). Similar effects were observed during activation by collagen, but not during PMA activation. It was concluded that the GPIIb/IIIa complex was intimately related to a part of the Ca^{2+} influx during the activation of platelets.

Glycoprotein GPIIb/IIIa; intracellular free Ca^{2+}; Thrombin; Collagen; Platelet

1. INTRODUCTION

An increase in intracellular free Ca^{2+} level, $[Ca^{2+}]_i$, is an important process in platelet activation. This increase in $[Ca^{2+}]_i$ might be caused by mobilization from internal Ca^{2+}-storage sites and/or influx from the extracellular medium. Recently, inositol 1,4,5-trisphosphate, a hydrolytic product from phosphatidylinositol 1,4-bisphosphate, has been suggested to act as a second messenger for the release of Ca^{2+} from intracellular storage sites [1]. The released Ca^{2+} returned by a Ca^{2+}-pumping system to internal compartments for storage [2–4]. On the other hand, the mechanism of Ca^{2+} flux across the platelet plasma membrane remains to be fully clarified; Doyle and Ruegg [5] suggested that the platelet plasma membrane lacks Ca^{2+} channels. Furthermore, several investigators have failed to find Ca^{2+}-ATPase in platelet plasma membranes [2,4].

Platelet membrane GPIIb and GPIIIa form a calcium-dependent heterodimer complex that contains binding sites for fibrinogen [6–9]. It is also thought to be a Ca^{2+}-transport system across platelet plasma membranes in resting platelets [10]. To clarify the role of GPIIb/IIIa in Ca^{2+} influx in human platelets under physiological conditions in a stirred medium, we employed aequorin as a $[Ca^{2+}]_i$ indicator. Aequorin, which is a photoprotein of molecular mass 20 kDa, binds Ca^{2+} and emits blue light [11,12], and the light emission of aequorin is not quenched by platelet aggregation. Here, we examined the effects of TM83 and a synthetic peptide, GRGDSP, on $[Ca^{2+}]_i$ changes in stimulated platelets and analyzed the role of GPIIb/IIIa complex in these changes.

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2. MATERIALS AND METHODS

Washed human platelets were prepared and then loaded with aequorin by using DMSO as in [13]. The aequorin-loaded platelets were suspended to about 3×10^5 platelets/μl in Hepes-buffered saline (140 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 5.6 mM dextrose, 0.1% bovine albumin, 3.8 mM Hepes, pH 7.5). The platelet suspension was preincubated with 1 mM $CaCl_2$ for 5 min or 1 mM EGTA for 30 s in some experiments, and then incubated with TM83 (20 μg/ml) [15], mouse IgG (20 μg/ml) or GRGDSP (100 μM) [16,17] at 37°C for 3 min. Aggregation was initiated by the addition of thrombin (final concentration, 0.05 or 0.1 U/ml), collagen (5 μg/ml) or PMA (32 nM) to the platelet suspension in a platelet ionized calcium aggregometer (PICA, Chrono-Log, PA) with continuous stirring. The increase in $[Ca^{2+}]_i$ was calculated according to Johnson et al. [14].

Aequorin was purchased from Chrono-Log, α-thrombin and phorbol 12-myristate 13-acetate (PMA) from Sigma, collagen from Hormon-Chemie, mouse IgG from Cappel, and the synthetic peptide Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP) from Peninsula. The anti-GPIIb/IIIa monoclonal antibody TM83 (subclass IgG) was prepared by hybridization. TM83 immunoprecipitated both GPIIb and IIIa from ^{125}I-surface labeled platelets. Crossed immunoelectrophoresis of platelets against anti-whole platelet antibody with ^{125}I-TM83 in the intermediate gel revealed that TM83 radioactivity was incorporated into both GPIIIa and the GPIIb/IIIa complex [15]. TM83 was purified from mouse ascitic fluid by protein A-Sepharose chromatography.

3. RESULTS

As shown in fig.1a, an increase in luminescence was observed immediately after the addition of thrombin in the control experiment (IgG). There was a small peak or a shoulder following the first peak. Pretreatment of the platelets with TM83 eliminated the second peak of $[Ca^{2+}]_i$ and decreased the height of the first. A similar inhibitory effect was obtained on pretreatment with GRGDSP (fig.1d). When EGTA was added in the suspension, the first peak decreased to about 50% of the control, and the second peak was abolished

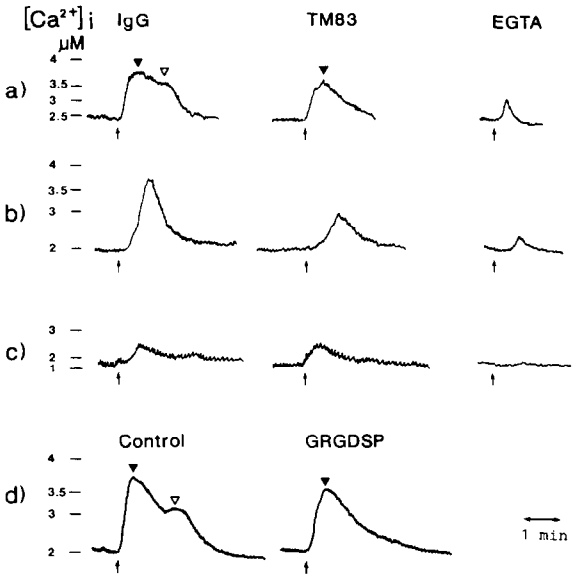

Fig.1. Effects of TM83, GRGDSP and EGTA on luminescence signals induced by various agonists. Platelets were preincubated with IgG (20 μg/ml), TM83 (20 μg/ml), GRGDSP (100 μM) or 0.9% NaCl (control) for 3 min or 1 mM EGTA for 30 s, and then various agonists were added. Arrows show the addition of agonists. (a) 0.1 U/ml thrombin, (b) 10 μg/ml collagen, (c) 32 nM PMA, (d) 0.05 U/ml thrombin. (▼) First peak, (▽) second peak.

(fig.1a). On the other hand, the increase in $[Ca^{2+}]_i$ induced by collagen or PMA did not show such a pattern of two peaks (fig.1b,c); the addition of EGTA reduced the $[Ca^{2+}]_i$ increase induced by collagen and PMA. TM83 reduced the increase in $[Ca^{2+}]_i$ induced by collagen, but not that by PMA. Table 1 summarizes the effects of TM83 on the increases in $[Ca^{2+}]_i$ and aggregation induced by thrombin, collagen and PMA. TM83 significantly inhibited platelet aggregation by these agonists. TM83 reduced the $[Ca^{2+}]_i$ increases induced by thrombin and collagen to 80 and 60% of the control, respectively, but not that by PMA. Fig.2 shows the effects of TM83 on changes in $[Ca^{2+}]_i$ accompanying aggregation induced by thrombin. Thrombin-stimulated platelet aggregation was gradually inhibited by increasing concentrations of TM83. 10 μg/ml of TM83 reduced thrombin-induced platelet aggregation to only 60% of the control value, while it completely eliminated the second peak of $[Ca^{2+}]_i$ increase.

Table 1

Summary of the effects of TM83 on increases in $[Ca^{2+}]_i$ and aggregation induced by various agonists

Reagent		IgG (20 μg/ml)	TM83 (20 μg/ml)
Thrombin	Δ $[Ca^{2+}]_i$ (μM)	1.33 ± 0.53	1.07 ± 0.48
(0.05 U/ml, n = 7)	aggregation (%)	53 ± 3.1	19 ± 7.0[a]
Collagen	Δ $[Ca^{2+}]_i$ (μM)	0.93 ± 0.14	0.56 ± 0.11
(5 μg/ml, n = 8)	aggregation (%)	42 ± 7.7	14 ± 3.9[a]
PMA	Δ $[Ca^{2+}]_i$ (μM)	0.46 ± 0.04	0.43 ± 0.06
(32 nM, n = 6)	aggregation (%)	29 ± 8.7	8 ± 1.4

[a] $p < 0.05$, significantly different from pretreatment with IgG

Δ $[Ca^{2+}]_i$, difference between resting $[Ca^{2+}]_i$ and the first peak of $[Ca^{2+}]_i$ induced by various agonists; Agg., transmittance change 3 min after the addition of agonists. Values are means ± SE

To investigate whether aggregation itself increases $[Ca^{2+}]_i$ in platelets, increases in $[Ca^{2+}]_i$ of platelets induced by the above agonists were observed with or without stirring (fig.3). Values of thrombin- and PMA-induced increases in $[Ca^{2+}]_i$ were 1.23 μM (n = 2) and 0.52 μM (n = 4), respectively, in stirred mixtures. Both increases were reduced to about 80% for the non-stirred states (fig.3). The second peak induced by thrombin or

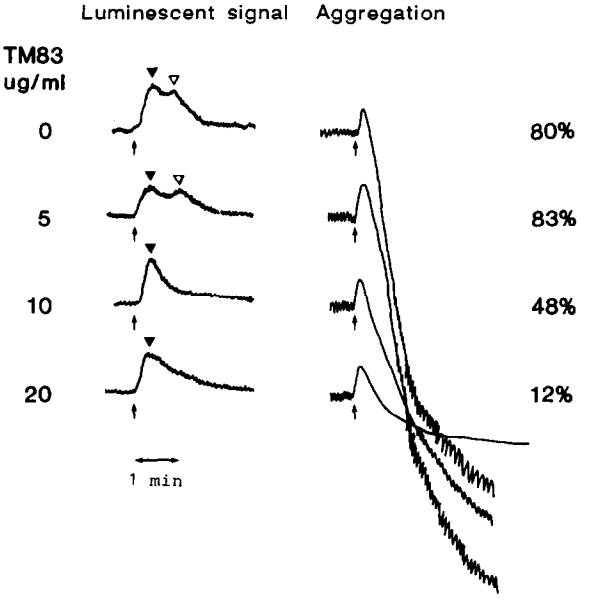

Fig.2. Effects of TM83 concentration on luminescence signals and aggregation induced by 0.05 U/ml thrombin. The degree of aggregation was calculated from the percent transmittance change after 3 min. Arrows indicate additions of thrombin. (▼) First peak, (▽) second peak.

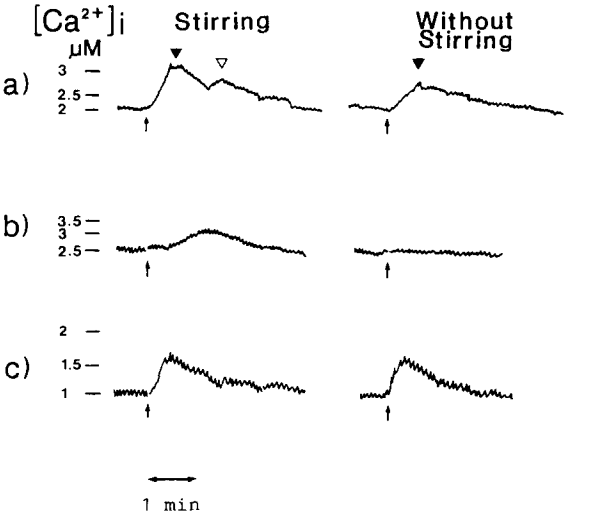

Fig.3. Effects of stirring conditions on luminescence signals induced by various agonists. Platelets in the cuvette were stirred continuously in the PICA before addition of agonists, then stirring was stopped or continued. (a) 0.05 U/ml thrombin, (b) 10 μg/ml collagen, (c) 32 nM PMA. (▼) First peak, (▽) second peak.

the first peak by collagen was lost in the non-stirred state.

4. DISCUSSION

Using the DMSO method [13], aequorin-loaded platelets exhibited two peaks or a shoulder pattern of $[Ca^{2+}]_i$ after stimulation by thrombin. When the platelets were stimulated by collagen or PMA, the second peak was absent. The presence of a second peak was not reported by others using a different loading method such as that of Johnson et al. [14]. TM83, a monoclonal antibody against GPIIIa that recognizes the GPIIb/IIIa complex, inhibited collagen-, ADP- or thrombin-induced aggregation and fibrinogen binding, but did not inhibit ATP secretion induced by thrombin [15]. GRGDSP, which is known to inhibit fibrinogen binding to ADP- or thrombin-stimulated platelets, was reported to inhibit platelet aggregation but not ATP secretion induced by thrombin [16,17]. In platelets, TM83 and GRGDSP reduced the first peak and eliminated the second peak of $[Ca^{2+}]_i$ induced by thrombin (fig.1, table 1). These effects were specific to GPIIb/IIIa since a monoclonal antibody to GPIb (TM60) [18] did not eliminate the second peak of $[Ca^{2+}]_i$ induced by thrombin (not shown). The second peak of $[Ca^{2+}]_i$ induced by thrombin was eliminated in the absence of aggregation under the non-stirred state (fig.3), suggesting that aggregation itself might be the triggering factor for the development of the second peak. However, a low concentration of TM83 partially inhibited thrombin-induced aggregation, but completely abolished the second peak of $[Ca^{2+}]_i$ (fig.2). This result indicates that the intact GPIIb/IIIa complex might be essential for the development of the second peak. Since the first peak, though reduced in height, still remained in the presence of 1 mM EGTA, it consists of the sum of Ca^{2+} mobilization and Ca^{2+} influx. Using quin2-loaded platelets, Powling and Hardisty [19] reported that anti-GPIIb/IIIa monoclonal antibodies reduced the $[Ca^{2+}]_i$ increases stimulated by several agonists including ADP, PAF and sodium arachidonate. They postulated that the effect might be due to steric hindrance of a Ca^{2+} channel closely adjacent to the GPIIb/IIIa complex, because Glanzmann's thrombasthenic platelets showed normal $[Ca^{2+}]_i$ responses to ADP stimulation. In contrast, we demonstrated that not only the monoclonal antibody TM83, but also the synthetic peptide GRGDSP had the same inhibitory effect on $[Ca^{2+}]_i$ responses to thrombin. Since GRGDSP binds to only the GPIIb/IIIa complex as does TM83, the former inhibitory effect cannot be explained by a mechanism involving steric hindrance because of its low molecular mass.

The collagen-induced increase in $[Ca^{2+}]_i$ was also mediated by GPIIb/IIIa. Moreover, aggregation itself was necessary for a collagen-induced increase in $[Ca^{2+}]_i$, since the peak of $[Ca^{2+}]_i$ was completely lost without stirring (fig.2). However, PMA activation, which directly activated protein kinase C [20], was not mediated through GPIIb/IIIa and aggregation.

According to the results, the mechanisms of Ca^{2+} influx into platelets can be classified into GPIIb/IIIa-dependent and independent pathways. Moreover, the presence of aggregation markedly influenced the $[Ca^{2+}]_i$ induced by thrombin and collagen. A possible role for the GPIIb/IIIa complex in Ca^{2+} influx can be postulated: the binding of fibrinogen to GPIIb/IIIa and/or the incorporation of GPIIb/IIIa into the cytoskeleton in aggregating platelets [21,22] might serve to open a pathway for Ca^{2+} influx in thrombin- and collagen-induced activation.

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REFERENCES

[1] O'Rourke, F.A., Helenda, S.P., Zavoico, G.B. and Feinstein, M.B. (1985) J. Biol. Chem. 260, 956–962.
[2] Dean, W.L. and Sullivan, D.M. (1982) J. Biol. Chem. 257, 14390–14394.
[3] Dean, W.L. (1984) J. Biol. Chem. 259, 7343–7348.
[4] Fischer, T.H., Campbell, K.P. and White, G.C. II (1985) J. Biol. Chem. 260, 8996–9001.
[5] Doyle, V.M. and Ruegg, U.T. (1985) Biochem. Biophys. Res. Commun. 127, 161–167.
[6] Nachman, R.L. and Leung, L.L.K. (1982) J. Clin. Invest. 69, 263–269.

[7] Jennings, L.K. and Phillips, D.R. (1982) J. Biol. Chem. 257, 10458–10466.
[8] Gogstad, G.O., Brosstad, F., Krutnes, M.B., Hagen, I. and Solum, N.O. (1982) Blood 60, 663–671.
[9] Brass, L.F. and Shattil, S.J. (1984) J. Clin. Invest. 73, 626–632.
[10] Brass, L.F. (1985) J. Biol. Chem. 260, 2231–2236.
[11] Shimomura, O., Johnson, F.H. and Saiga, Y. (1962) Aequorea. J. Cell Comp. Physiol. 59, 223–239.
[12] Prasher, D., McCann, R.O. and Cormier, M.J. (1985) Biochem. Biophys. Res. Commun. 126, 1259–1268.
[13] Yamaguchi, A., Suzuki, H., Tanoue, K. and Yamazaki, H. (1986) Thromb. Res. 44, 165–174.
[14] Johnson, P.C., Ware, J.A., Cliveden, P.B., Smith, M., Dvorak, A.M. and Salzman, E.W. (1985) J. Biol. Chem. 260, 2069–2076.
[15] Yamamoto, N., Kitagawa, H., Tanoue, K. and Yamazaki, H. (1987) Acta Haematol. Jap., in press.
[16] Ginsberg, M., Pierschbacher, M.D., Rouslahti, E., Marguerie, G. and Plow, E. (1985) J. Biol. Chem. 260, 3931–3936.
[17] Plow, E.F., Pierschbacher, M.D., Ruoslahti, E., Marguerie, G.A. and Ginsberg, M.H. (1985) Proc. Natl. Acad. Sci. USA 82, 8057–8061.
[18] Yamamoto, N., Kitagawa, H., Tanoue, K. and Yamazaki, H. (1985) Thromb. Res. 39, 751–759.
[19] Powling, M.J. and Hardisty, R.M. (1985) Blood 66, 731–734.
[20] Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. and Nishizuka, Y. (1982) J. Biol. Chem. 257, 7847–7851.
[21] Phillips, D.R., Jenning, L.K. and Edwards, H.H. (1980) J. Cell Biol. 86, 77–86.
[22] Wheeler, M.E., Gerrard, J.M. and Carroll, R. (1985) J. Cell Biol. 101, 993–1000.